# IDENTIFICATION OF HUMAN PAPILLOMA VIRUS (HPV) IN SALIVA OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) - SEROPOSITIVE ADULTS SIX MONTHS POST HAART

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*In partial fulfillment for the Degree of*

**MASTER OF DENTAL SURGERY**


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# CERTIFICATE

This is to certify that this dissertation titled "**IDENTIFICATION OF HUMAN PAPILLOMA VIRUS (HPV) IN SALIVA OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) - SEROPOSITIVE ADULTS SIX MONTHS POST HAART"** is a bonafide dissertation performed by **SREEJA. C** under our guidance during the postgraduate period 2008-2011.

This dissertation is submitted to THE TAMILNADU DR. M.G.R MEDICAL UNIVERSITY, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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The number of people living with HIV globally is 33.4 millions[1]. Systemic and oral lesions in HIV infection reflect the immune status of the patients and these lesions are important not only for the morbidity that they cause, but also for their diagnostic value in monitoring the immune status of the patient. The occurrence of oral lesions indicate a great susceptibility for opportunistic infections and a great possibility for a rapid progression to AIDS[2,3,4]

The introduction of antiretroviral therapy (ART), especially combination therapy –has led to the reduction of opportunistic infections, mortality and morbidity in HIV infected patients. Highly active antiretroviral therapy (HAART) is defined as an antiretroviral regimen containing a minimum of 3 drugs, nucleotide reverse transcriptase inhibitor (NRTI), protease inhibitor and non nucleotide reverse transcriptase inhibitor (NNRTI).[5]

HPV infection is recognized as the most common sexually transmitted infection. The association between HPV and cervical cancer has been recognized[6.] In humans more than 70 types have been described (*Zur Hausen*, 1996). Mucosal and genital HPV consisting of about 30 types ,are divided into low risk (HPV 6,11,42,43 and 44) and high risk (HPV 16,18,31,33,35,45,51,52 and 56), according to their presence in malignant lesions of the

cervix *(Howy et al 1994)*[7]. There are circumstantial evidence suggesting that high risk HPVs are involved in oral carcinogenesis[8].

Estimates of oral HPV prevalence is variable due to methodological or population differences and ranges from 9.2 – 18.8%[9]. When HPV lesions are present in association with HIV infection, some patients experience a rather sudden occurrence and growth of new lesions in the mouth concurrently with deterioriation of the immune status. The oral warts caused by HPV are usually 1-3mm in diameter, and may be located anywhere in the mouth, but most often on the labial or buccal mucosa or the gingiva[10].

Prevalence of HPV associated oral condylomas have reportedly increased in HIV infected individuals since the introduction of HAART[11] and oral warts due to HPV also have dramatically increased in the HAART era[12]. Greenspan et al studied the effect of HAART on frequency of oral warts and found that HIV infected patients on HAART were six times more likely to develop warts than patients not on HAART. They also postulated that the functionally incomplete reconstitution of the immune system, observed in patients initiated on HAART could alter its effectiveness with regard to different potentially pathogenic microorgansisms and may probably lead to the development of oral warts in the context of an overall reduction of opportunistic infections[13].

Our initial studies reported absence of clinical oral warts in HIV patients on HAART[14,3] Similarly in our earlier longitudinal study in 2007, on oral lesions in HIV positive patients with and without HAART, HPV induced lesions were not encountered[15].

It has been stated that HPV frequently establishes a subclinical or latent infection in the oral cavity, which could serve as a reservoir for HPV transmission and future disease. It has also been shown that saliva can be used to study the epidemiology of HPV infection of the oral cavity in HIV infected patients and by using saliva, oral HPV was detected equally in those on HAART and those not on HAART and those who did not have oral warts clinically. It has also been documented that HPV infection was detected more frequently on those on effective HAART compared to those on clinically ineffective HAART[11].

A cross sectional study was done at our centre (Ragas Dental college and Hospital, Chennai) between 2009-2010 to determine HPV genome in the saliva of HIV seropositive patients naive to HAART, and those who fulfilled the criteria for initiation of HAART. L1 consensus primer which was common to all HPV was used in this study (by polymerase chain reaction). HPV was not detected in this study group[16].

In the present study we followed the above patients 6 months after the commencement of HAART for detection of any HPV genome by PCR technique using L1 consensus primer to assess any latent infection by HPV.

**AIM:**

To identify Human Papilloma Virus in Saliva of Human Immunodeficiency Virus (HIV) - Seropositive adults 6 months post HAART.

**OBJECTIVES:**

To detect the presence of Human Papilloma Virus (HPV) in saliva of

- HIV seropositive adults on six months of highly active anti retroviral therapy (HAART)
- HIV seronegative adults.

**HYPOTHESIS:**

Initiation of HAART in HIV seropositive individuals does not influence the salivary HPV status.

**STUDY DESIGN**

A prospective study was done to ascertain oral HPV status in the saliva by using PCR to amplify the late gene L1 of HPV genome

**STUDY SUBJECTS:**

**GROUP I**: Ten HIV seropositive patients who were on HAART ≥ 6 months for whom HPV status was ascertained prior to initiation of HAART

**GROUP II**: Ten HIV seronegative individuals.

**STUDY SETTING**

- Saliva samples were collected from patients attending the out patient wing of YRG CARE, VHS, Chennai
- Study was conducted at Ragas Dental College and Hospital, Chennai

**STUDY PROCEDURE**

- Ethical clearance was obtained from the institutional review board of Ragas Dental College and from YRGCARE
- Informed consent was obtained from the patient
- Demographic details of the patient were recorded
- A thorough oral examination was done and findings were recorded in prestructured case sheets
- SAMPLE COLLECTION
    - The patient was asked to sit on a chair, and after spitting any residual saliva present in his mouth, he was

asked to spit into a sterile container every minute for 10 minutes to collect unstimulated whole saliva. *(Journal of American academy of dermatology 2000, 43: s18-26)*

- 5ml of unstimulated whole saliva was collected in the morning after breakfast (9:00 am – 11:00 am) from the study subjects (to avoid diurnal variation)
- Buccal mucosal scrapping was collected from 5 patients of group I selected randomly
- The sample was immediately transported to the research laboratory at Ragas dental college and hospital and stored at $-70^0$C until DNA extraction

## ARMAMENTARIUM

1. For patient examination and sample collection
    - Gloves and mouth masks
    - Sterile mouth mirror and straight probe
    - 50ml sample collection containers
    - Normal saline
    - Wooden spatula
2. For DNA extraction
    - Microcentrifuge tubes
    - Positive displacement pipettes
    - DNA extraction kit
    - Thermostat
    - Cooling centrifuge
3. DNA amplification
    - Thermal cycler
    - MY09/11 L1 consensus primer
    - Housekeeping gene primers
    - PCR master mix
    - Positive control: HeLa cell line DNA
4. Gel electrophoresis
    - Agarose
    - TAE (Tris Acetate EDTA)
    - Ethidium bromide
    - Horizontal electrophoresis unit

- Microwave
- Gel Dock system

**DNA EXTRACTION**

Saliva samples were taken from the deep freezer and kept for thawing till they reached the room temperature.

- 1500µl of saliva was taken in a 1.5ml micro centrifuge tube.
- The tube was Centrifuged for 5 minutes at 13000rpm.1200µl of the supernatant was discarded from it.
- 900µl of Real Biotech Corporation (RBC) lysis buffer was added to it and mixed by inversion method.
- The tube was incubated for 5 minutes at room temperature.
- It was then centrifuged at 1300 rpm for 5 minutes and the supernatant was discarded.
- 100 µl RBC lysis buffer was added to resuspend the cell pellet.
- 200µl GB buffer was then added to the tube and mixed by vortex.
- The mixture was incubated at room temperature for 10 minutes until the sample lysate became clear. During incubation, the tube was inverted every 3 minutes.
- The required quantity of elution buffer (200µl / sample) was preheated in a 70° C water bath (for DNA elution).
- 200µl of ethanol (96-100%) was added to the sample lysate and was mixed immediately by vortexing for 10 seconds.

- GD (a tube with a sieve) column was placed in 2ml collecting tube.
- Total mixture (including any precipitate) from the previous step was added to the GD column.
- The cap of the column was closed and centrifuged at 13000rpm.
- 200µl of GB buffer was added to it and centrifuged at 6000rpm for 5 minutes.
- 400 µl of W1 buffer was added into the GD column and centrifuged at 13000 rpm for 30 seconds.
- The flow-through was discarded and GD column was placed back in the 2ml collecting tube.
- 600 µl of wash buffer was added into the GD column.
- It was then centrifuged at 13000 rpm for 30 seconds, the flow-through was then discarded and the GD column was placed back in the 2ml collecting tube.
- It was then centrifuged at 13,000 rpm for 5 minutes to dry column matrix.
- Dried GD column was then transferred into clean 1.5 ml micro-centrifuge tube.
- 100 µl of preheated elution buffer was added into the centre of column matrix.
- It was then allowed to stand at room temperature for 5 minutes until elution buffer is absorbed by the matrix and

centrifuged at 13000 rpm for 30 seconds to elute purified DNA.

- The extracted DNA was stored at - 4 ° C till it was further used.

**DNA AMPLIFICATION USING PCR**

- The primers PC04 and GH20 were selected to amplify the β chain of haemoglobin to validate the PCR amplification of genomic DNA.
- PC04 and GH20 primers yielded a product of 268 base pairs.
- MY09/11 primers were used to amplify the L1 region of the HPV genome.
- MY09/11 primers yielded a product of 450 base pairs. The primer sequences were as follows:

**β globin primer**

**PC04 :** CAA CTT CAT CCA CGT TCA CC

**GH20 :** GAA GAG CCA AGG ACA GGT AC

**L1 consensus primers for HPV:**

**MY 09 :** CGT CCM ARR GGA WAC TGA TC

**MY 11 :** GCM CAG GGW CAT AAY AAT GG

- The primers, DNA extracted from the sample, master mix, positive control and DEPC water were taken out from the deep freezer and kept for thawing.

- The laminar flow was cleaned with acetone and UV light was switched on for 30 minutes.
- 50 µl reaction mixtures were prepared in the laminar flow unit using positive displacement pipettes as follows:

| | **Positive control** | **Sample** | **Negative control** |
|---|---|---|---|
| **Master mix** | 25 µl | 25 µl | 25 µl |
| **MY 09 primer** | 1 µl | 1 µl | 1 µl |
| **MY 11 primer** | 1 µl | 1 µl | 1 µl |
| **PC 04 primer** | 1 µl | 1 µl | 1 µl |
| **GH 20 primer** | 1 µl | 1 µl | 1 µl |
| **DNA** | 6 µl of HeLa DNA | 6 µl sample DNA | 6 µl of DEPC water |
| **DEPC water** | 15 µl | 15 µl | 15 µl |

Positive and negative controls were included in each PCR amplification cycle. The final 50µl reaction mixture was amplified in a thermal cycler. The amplification programme was as follows:

| **Number of cycles** | **Temperature** | **Time** |
|---|---|---|
| 1 | Initial denaturation 96 $^{o}$c | 5 minutes |
| 30 | Denaturation 94 $^{o}$c<br>Annealing 63 $^{o}$c<br>Synthesis 72 $^{o}$c | 1 minute<br>1 minute<br>1 minute |
| 1 | Final extension 72 $^{o}$c | 5minutes |

## GEL ELECTROPHORESIS

1.2% agarose gel was prepared using 1xTAE buffer and agarose powder. 0.72 grams of agarose powder was weighed and transfered into a clean conical flask, washed with deionised water. To this 60ml of 1xTAE buffer was added and it was heated in a microwave oven for one minute. The conical flask was taken out after every 20 seconds and swirled to mix. It was then allowed to cool.

Meanwhile, the gel electrophoresis tank, boat and comb were washed using deionised water. The ends of the gel boat were sealed using adhesive tape. 15µl Ethidium bromide was added to the agarose solution, it was swirled well and poured into the gel boat having the gel comb. The agarose was allowed to cool and gel. The gel comb was then removed slowly to leave behind wells in the gel. The adhesive tape was removed and the boat was placed in the tank. 1xTAE was poured into the tank so as to immerse the gel completely.

2µl of loading dye was mixed with 8µl of 100 base pairs DNA ladder and was loaded into the well in the gel. 8µl of sample, positive control and negative control were mixed with 2µl of loading dye separately and loaded into the wells. The gel tank was connected to the electrophoresis unit which delivered 100v electric current for 45 minutes. The electrophoresis unit was disconnected and the gel was taken out and placed on an UV illuminator and

visualised using Gel Dock system. The images of the gel was captured and stored.

Positive control showed a beta globin band corresponding to 268 base pairs and a HPV band corresponding to 450 base pairs of DNA ladder. The negative control did not have any band. All Samples showed a beta globin band whereas the HPV band was not present.

- Datas were entered and statistically analyzed by using SPSS$^{TM}$ software (Version 10.0.5)
- Descriptive analysis was used to analyze the variables
- Paired - t test was used for the variables CD4 and Hb count (to compare between pre HAART and post HAART
- p value $\leq 0.05$ was considered to be statistically significant

## Human Papilloma Virus Genome

**Stephen K Tyring (2000)**[17] reviewed the epidemiology, pathogenesis and host immune response of human papilloma virus and described the HPV genome as a closed ,double stranded 8 Kb DNA molecule that comprises early and late gene clustered in separate regions. Early gene codes for protein involved in viral DNA replication, transcription control and cellular transformation. Late gene encodes the major viral capsid protein (L1) and minor capsid protein (L2). Between these two regions is an upstream regulatory region (URR) also known as long control region. This non coding region contains promoters for E6 common to all HPVs but also one or more specific promoters in the URR.

**Lehtinen M and J Paavonen (2001)**[18] In their review on the efficacy of preventive human papilloma virus vaccination described that the DNA of Human Papilloma virus consists of approximately 8000 base pairs and comprises a long control region (LCR) and open reading frames (ORF) all of which are transcribed at different stages of the natural infection. Viral capsid consists of late L1 (major) and L2 (minor) proteins but the latter is not necessary for capsid assembly. Seven early ORFs (E1 to E8) specify regulatory proteins to participate in viral transcription (E2, E1), replication (E1, E2), interaction between viral and cellular proteins (E2-E7) and malignant transformation (E6, E7).

**S Nair and MR Pillai (2005)**[7] in their review on relevance of Human Papilloma Virus disease mechanisms to oral and cervical cancers , explained the genome of these viruses as a double stranded DNA molecule of about 8000bp. They also described the three genomic regions as a late region (L), an early region (E), and a long control region (LCR). The early gene $E_1$ and $E_2$ encodes for the protein involved in viral DNA replication and controls viral transcription. The products of gene $E_6$ and $E_7$ are essential in HPV induced cell immortalization and transformation and the late gene $L_1$ and $L_2$ encodes for the viral capsid protein.

**HPV in normal oral and cervical mucosa**

**Badaracco G, Venuti A, Di Lonardo *et al* (1998)**[19] in their study, analysed cervical smears and oral swabs from 29 women. HPV-DNA was detected in 34.5% cervical smears and 37.9% of oral swabs. HPV-16 was present in 53.8% and was the most prevalent genotype followed by HPV-6, which was present in 34.6% of the positive samples. Other types were rarely detected. Concurrent genital and oral HPV infection was seen in 5 patients (17.24%).

**Kurose K, Terai M, Soedarsono N and Rabello D (2004)**[20] investigated the incidence of HPV infection in normal oral mucosa. They also followed the HPV-positive participants of this cohort study approximately 2.5 years after the first sample collection. Of the 662 samples analyzed, HPV was detected only in 0.6% of samples, suggesting that oral HPV infection is uncommon. In the

follow-up survey, the HPV71 and HPV12-positive participants again tested positive, while HPV DNA was not detected in the HPV16 and HPV53-positive participants.

**Rojas M, Schlecht N and Nucci-Sack A (2007)[21]** *et al* examined the prevalence of cervical, anal and oral HPV in sexually active adolescents. They observed that infection with low-risk HPV types most associated with anogenital warts and condylomas was 40.6% in cervical, 30.4% in anal and 7.5% in oral mucosa. In addition infection with oncogenic HPV types was found to be as high as 40.6% in cervical, 32.6% in anal, and 4.3% in oral mucosa.

**Castro T, Filho I, Nascimento V and Xavier S (2009)[22]** investigated the presence of HPV DNA in the oral and genital mucosa of women with HPV genital infection, using the PCR**.** Swabs were obtained from the oral and genital mucosa of 30 women with histopathologically confirmed HPV genital infection. HPV DNA was not detected in any oral swabs, while in the genital tract, HPV was detected in 17 (57%) of the 30 patients. They suggested that genital HPV does not seem to be a predisposing factor for the oral infection.

**HPV in normal, premalignant and malignant oral lesions**

**Schwartz S, Daling J, Doody D *et al* (1998)[23]** conducted a case-control study to determine if oral-cancer risk is related to HPV infection. HPV DNA was detected in 26% of tumours using PCR.

16.5 % of tumours contained HPV16 DNA. The Odds Ratio for HPV type 16 seropositivity was 2.3 for all oral SCCs and 6.8 for oral SCCs containing HPV type 16 DNA. Their findings suggested that HPV16 infection may contribute to the development of a small proportion of OSCC.

**Elamin F, Steingrimsdottir H, Warnakulasuriya S *et al* (1998)[24]** evaluated the role of HPV in oral neoplasms using nested PCR. HPV 16 and HPV 18 were the only types detected in all tumors by HPV typing. HPV DNA was detected in 33% of premalignant lesions suggesting a possible role of HPV in oral epithelial dysplasia.

**Bouda M, Gorgoulis V, Kastrinakis G *et al* (2000)[25]** examined HPV infection in oral hyperplasia, dysplasia and SCC as well as in normal mucosal specimens. HPV DNA was detected in 91% of pathological samples but not in normal specimens. Of the HPV DNA positive samples, 98% were infected with high-risk type, whereas only 2% were infected with low-risk type of HPV. These findings suggest that high-risk HPV types are involved in oral carcinogenesis.

**Jimenez C, Correnti M, Salma N *et al* (2000)[26]** investigated the presence of HPV infection in clinically normal and benign oral epithelial lesions. Biopsy specimens of 40 patients with benign epithelial lesions and 20 patients with clinically normal mucosa were subjected to PCR analysis using MY09/11 primers. Viral DNA

was detected in 59% of oral benign lesions and 10% of biopsy specimens of normal mucosa.

**Ha P, Pai S, Westra W *et al* (2002)** [27] evaluated the role of HPV 16 in the progression of oral head and neck cancer. They quantified the HPV 16 DNA in pre-malignant and malignant lesions using quantitative PCR. HPV DNA was detected in 0.98% of 102 premalignant lesions and 2.9% of invasive oral cancers. 14 of the 18 invasive tumors, previously found to be HPV positive by methods other than PCR, were positive by quantitative PCR. This suggested that HPV 16 may not play a major role in malignant progression of premalignant lesions.

**Sugiyama M, Bawal U, Dohmen T *et al* (2003)**[28] studied the association between HPV and oral carcinogenesis. They analysed specimens of normal mucosa, epithelial dysplasia and OSCC and OSCC cell lines, using PCR. HPV16 DNA was detected in 61% of epithelial dysplasia specimens, 36% of normal and 35% of OSCC tissues. HPV18 DNA was not detected in any of these cases, suggesting that HPV16 may be correlated with an early stage of oral carcinogenesis. They suggested that even if HPV16 plays a role, it just could be an initiator of proliferation and not a requisite for maintenance and progression of malignant stage. HPV cannot replicate in proliferating cultured cells that do not undergo differentiation and hence HPV DNA may be lost in later stages.

**Patrick K and Califano J (2004)** [29] reviewed the role of HPV in oral carcinogenesis. They stated that although epidemiological and molecular data provides evidence of high-risk HPV presence in oral pre-malignant and malignant lesions, it is unlikely in most cases. HPV may be a contributing factor in subset of oral malignancies, but is not a necessity in all cases as in cervical cancer.

**HPV in HIV sero positive patients**

**Cappiello G, Garbuglia A, Salvi L *et al* (1997)**[30] assessed the association between different HPV genotypes, HIV infection and cervical squamous intra-epithelial lesions (SIL) in 236 HIV seropositive women. The prevalence of HPV 16 and 18 was similar among both HIV-positive and HIV-negative women. HIV positive women showed a wider spectrum of HPV genotypes.

**Palefsky J, Holly E, Ralston M and Jay N (1998)**[31] studied the presence of anal HPV infection in 346 HIV positive and 262 HIV negative homosexual and bisexual men and detected anal HPV DNA in 93% of HIV-positive and 61% of HIV-negative men by polymerase chain reaction. HPV-16 was the most common type of HPV in both the groups. They also observed that lower CD4+ cell levels were associated with higher levels of oncogenic HPV types.

**Piketty C, Darragh M, Da Costa M *et al* (2003)**[32] conducted a cross-sectional study to assess the prevalence of anal HPV in HIV positive men. 46% of the 50 HIV positive heterosexual injection

drug users and 85% of the HIV positive men who have sex with men had anal HPV infection. They concluded that anal HPV infection may be acquired in the absence of anal intercourse in HIV-positive men.

**Pinto A, Baggio H and Guedes G (2005)**[33] described the common sexually transmitted viral diseases. These included HIV, genital warts, intra-epithelial lesions, genital squamous cell carcinomas and herpes simplex virus (HSV) infection. Women infected with HIV were found to be at a greater risk of being co-infected with HPV and are more prone to progression to persistence of HPV lesion.

**Shetty K, Chattopadhyay A and Leigh (2005)**[34] investigated and described the HPV types present in oral warts of HIV positive patients. DNA extracted from the 12 biopsy samples of patients who had a clinicopathologic diagnosis of oral warts, were subject to multiplex PCR assay. HPV was detected in 11 of the 12 orals warts. HPV 32 and HPV 7 were the only HPV types detected. HPV-32 was present in all subjects, whereas only one subject had a co-infection of HPV 32 and HPV 7.

**Sirera G, Videla S, Pinol N *et al* (2006)**[35] evaluated the prevalence of HPV in the anus, penis and mouth of men who have sex with men (MSM) and hetero-sexual men. PCR was used for assessing the prevalence of HPV DNA. A high HPV prevalence of 78%, 36% and 30% were noted in the anus, penis and mouth of HIV positive men

respectively. The findings support the suggestion that presence of HPV infection should be determined in any HIV infected male patient.

**Palefsky J (2006)**[36] in his review said that HIV-positive men and women are at an increased risk of anogenital and oral HPV infection. The risks for HPV-associated high-grade intra-epithelial neoplasia (IN) and cancer are also high. The prevalence of oral, anal, and cervical HPV infection in HIV-positive individuals compared with HIV-negative individuals increases with progressively lower CD4+ levels. Immune suppression plays a more prominent role in the earlier stages of HPV-associated disease.

**Fakhry C, D'Souza G, Sugar E *et al* (2006)**[37] compared the prevalence and type distribution of oral and cervical HPV infection in HIV positive women. They detected HPV DNA in oral rinse and vaginal lavage samples. In HIV positive and negative women, the oral HPV infection was 25.2% and 9% and cervical HPV infection was 76.9% and 44.9% respectively. One-third of HPV types detected in the cervix were not detected in the oral cavity of the same women. Most women with an oral HPV infection also had a cervical infection. This suggests that oral cavity is a significant reservoir for HPV, which may not be entirely independent of as cervical reservoir.

**Richter K, van Rensburg E, van Heerden W and Boy S (2008)**[38] harvested oral epithelium from buccal mucosa and lateral borders of the tongue and cervical samples from the endocervical area of 30 HIV infected women. Oral HPV was detected in 20% of the patients whereas genital HPV was found in 96.7% of the women, of whom only 14 had cytological abnormalities on Papanicolau smear. Women with CD4 counts less than 300 cells per ml had a significant risk of cervical HPV infection. Though HPV was detected in the oral cavity, only a limited correlation between oral and cervical HPV types was found.

**Singh D K, Anastos K and Hoover D R (2009)**[39] analysed **c**ytologic and HPV data available for 647 HIV-positive women and 188 HIV-negative women in Ruwanda. They collected cervicovaginal lavage specimens and tested them for 40 HPV types by a PCR assay. They found that the prevalence of HPV was higher in HIV-positive women than in HIV-negative women in all age groups. Among HIV-infected women, 69% were positive for one HPV type, 46% for a carcinogenic HPV type, and 10% for HPV-16. HPV prevalence was the highest in the HIV-positive women aged 25–34 years (75%) and then declined with age to 37.5% in those >55 years old. A higher prevalence of HPV and carcinogenic HPV were associated with lower CD4+ cell counts and increasing cytologic severity among HIV-positive women.

**Baumgarth N, Szubin R, Dolganov G, *et al* (2004)** [40] studied the expression of 166 genes in epithelial and sub-epithelial compartments of normal mucosa from HIV-infected and non-infected patients and of HPV32-induced oral warts from HIV infected patients. Changes in oral warts were strongly tissue substructure-specific. They suggested that HPV 32 establish infection by selectively enhancing epithelial cell growth and differentiation in the stratum spinosum and to evade the immune system by actively suppressing inflammatory responses in adjacent underlying tissues.

**Oral lesions in HIV infection**

**Greenspan D and Greenspan S (1996)** [2] reviewed the HIV related oral lesions most commonly encountered. Of these OC is the most common lesion. HIV related oral diseases may be grouped into lesions of fungal origin, lesions of bacterial origin, viral origin, auto immune and neoplastic conditions.

**Rangananthan K, Reddy B V R, Kumarasamy N *et al* (2000)**[14] studied the prevalence of oral lesions in 300 HIV patients of south-India. Oral lesions were seen in 72% of the patients. Gingivitis (47%) was the most common oral lesions followed by pseudo membranous candidiasis (PC) (33%), oral mucosal pigmentation (23%) and erythematous candidasis (EC) (14%). Other lesions that

were seen included periodontitis, angular cheilitis, oral ulcers, oral hairy leukoplakia (OHL) and oral sub mucous fibrosis.

**Ramirez-Amador V, Esquivel-Pedraza L, Sierra-Madero J *et al* (2003)**[41] studied the prevalence of oral lesions in 1000 HIV patients over a period of 12 years stratified into 4 periods (2 were before the introduction of HAART and the last 2 were during more established use of HAART). OC (31.6%) and OHL (22.6%) were the most common lesions whereas oral KS, HIV associated periodontal disease and oral non-Hodgkin's lymphoma was less frequent. During the course of the 4 study periods, the oral lesions decreased systematically. Except kaposi sarcoma (KS), all oral lesions strongly associated with HIV showed a significant decrease during the study period. There was no variation in the occurrence of salivary gland disease or HPV associated oral lesions. The patients with oral lesions which are strongly associated with HIV had lower median CD4+ cell counts. The decrease in oral disease was attributed to the use of prophylactic drugs and introduction of HAART.

**Ranganathan K, Umadevi K, Saraswathi T R *et al* (2004)**[3] studied the oral lesions and gender differences in a large cohort of 1000 HIV patients. Gingivitis (72.3%) was the most common oral lesion. Other oral lesions included periodontitis (33.2%), oral pigmentation (26.3%), OC (23.8%), angular cheilitis (7.9%), oral ulcers (3.3%), OHL (2.1%), leukoplakia (1.5%) and oral submucous fibrosis

(0.9%). The prevalence of oral lesions was more in males (88%) than females (82%). This difference in the occurrences was statistically significant.

**Leigh J, Kishore S and Fidel P (2004)**[42] reviewed the oral opportunistic infections and the role of mucosal immune function in HIV-positive individuals. They found that both systemic and local immunity were important for protection against oropharyngeal candidiasis. Recent investigations into the host responses associated with OHL and oral warts did not show any evidence of systemic or mucosal immune responsiveness.

**Inés María Bravo María Correnti, Laura Escalona et al (2005)**[4] studied the prevalence of oral lesions in HIV patients related to CD4 cell count and viral load in a Venezuelan population. They evaluated 75 HIV seropositive adult patients and found that 85% of patients showed associated oral lesions . oral candidasis constituted the most common lesion (61%) followed by oral hairy leukoplakia (34%), melanic hyper pigmentation (38%), papilloma (13%), lineal gingival erythema, apthous stomatitis (5%), Kaposi sarcoma (5%) and concluded that high viral load was strongly associated with the occurrence of oral lesions independent of CD4 cell count.

**Maeve M. Coogan John Greenspan and Stephen J. Challacombe (2005)** [43] discussed the importance of oral lesions as indicators of infection with HIV and as a predictor of progression of HIV disease

to acquired immune deficiency syndrome (AIDS). They described that oral manifestations are among the earliest and most important indicators of infection with HIV. Seven cardinal lesions, oral candidiasis, oral hairy leukoplakia, Kaposi sarcoma, linear gingival erythema, necrotizing ulcerative gingivitis, necrotizing ulcerative periodontitis and non hodgkins lymphoma were explained as strongly associated with HIV infection and have been identified internationally.

**Ranganathan K and Hemalatha R (2006)**[44] reviewed the literature on oral lesions affecting the HIV positive patients in developing countries. OC was the most common opportunistic infection in all continents. KS was limited to patients from Africa and Latin America. Salivary gland disease was more common in paediatric HIV subjects. A high prevalence of gingivitis and periodontitis were reported from India and Africa. Considerable regional prevalence of oral manifestations were highlighted.

**Sharma G, KM Pai, S Suhas et al (2006)**[45] assessed the prevalence of HIV related oral lesions in South India. They concluded that oral hairy leukoplakia showed a positive relationship with patients younger than 35 years. Oral candidasis can act as a marker of immune suppression

**Alan Grupioni Lourenco, Luiz Tadeu Moraes e (2008)**[46] studied the prevalence of oral lesions in 340 HIV infected individuals of

Brazil. The oral lesions encountered in their study were oral candidiasis (17.7%), angular chelitis (13.9%), hairy leukoplakia (11.8%) and oral ulcers (2.1%). They concluded that oral lesions inversely correlates with CD4 count and directly correlates with HIV viral load and suggested that oral lesions could be used as an alternative clinical marker for poor immune condition in HIV infected individuals.

**ORAL LESION IN HIV POST HAART**

**Andrea M, Frank P, Frank B and Peter R (2000)** [47]compared the prevalence of oral lesions in HIV-seropositive before and after the initiation of HAART. One hundred and three HIV serpositive patients (88 men and 15 women) who were on combination therapy for at least four weeks at the time of study were included . They re-evaluated the patients twice in an interval of six months each and the most frequent lesions were OHL and OC. The prevalence of oral lesions after the onset of HAART decreased significantly in their study but in four patients the immunological effects of therapy did not provide sufficient protection against HPV induced lesions.

**Eyeson J D, Flowers M T, Cooper D J *et al* (2002)** [48] determined the prevalence of oral lesions in HIV positive patient, predominantly on HAART. 49% of the patients had no detectable oral lesions. Oral lesions detected most frequently included OHL (9.9%), HIV associated periodontal diseases (9.9%) and OC (4.9%). Three patients (1.5%) had multiple papillomatous growths. This

study showed a low prevalence of HIV associated oral lesions and a change in the pattern of predominant lesions in the era of HAART. These lesions were more likely to be found in patients with advanced disease, patients not receiving therapy, or in patients in whom therapy was failing.

**Reznik D (2005)**[49] reviewed the HIV related oral conditions in HIV infected individuals. Common oral lesions in HIV patients included xerostomia, OC, OHL, periodontal disease, KS, HPV associated warts and oral ulcers. It was noted that the prevalence of oral lesions reduced from 47.6 to 37.5% after introduction of HAART.

**Flint S R, Tappuni A, Leigh J *et al* (2006)**[50] reviewed the literature on oral lesions in HIV patients and reported that the overall prevalence of HIV related oral disease decreases with HAART therapy. Lesions such as oral warts and salivary gland disease decreased with the use of HAART.

**Tappuni A and Fleming G (2001)** [51] compared the prevalence of oral lesions among 89 HIV infected patients who were on ART and 195 HIV infected patients not on ART. The prevalence of oral lesions was 30% in patients on any ART compared with 46% in patients not on ART.

**Sharma G, Pai KM, Suhas S *et al* (2006)**[45] evaluated the prevalence of oral lesions in HIV positive individuals. Oral manifestations were seen in 79% of patients. EC (44.5%) was the

most common lesion, followed by melanotic hyper pigmentation (34.6%), OHL (15.8%), linear gingival erythema (15.8%), PC (11.8%) and angular cheilitis (11.8%).

**Umadevi K, Ranganathan K, Pavithra S *et al* (2007)**[15] described oral lesions in hundred HIV positive patients initiated on highly active anti-retroviral therapy (HAART). A significant decrease in the occurrence of OC (from 24% to 8%) was seen in patients after initiation of HAART. The prevalence of oral melanin pigmentation in patients with CD4+ cell counts of >200 cells/µl increased from 14.8% to 43.8% after initiation of HAART.

**Greenspan D, Canchola J, MacPhail L *et al* (2001)**[13] investigated the change in prevalence of oral lesions after initiation of HAART in HIV patients. This retrospective study revealed a significant decrease in common oral lesions and increase in salivary gland disease. The prevalence of oral warts was 5% among patients not on any anti-retroviral medication, whereas it was 15% in patients on anti-retroviral therapy and 23% in patients on HAART.

**King M, Reznik D, O'Daniels C M *et al* (2002)**[12] conducted a case-control study to assess the oral warts among HIV sero-positive patients. The prevalence of oral warts among 2194 HIV seropositive patients was 2.6%. The HIV viral load was lower among patients with oral warts. An association between the use of HAART and oral warts was not seen.

**Cameron J, Marcante D, O'brien M *et al* (2005)[11]** assessed the impact of highly active anti-retroviral therapy (HAART) on oral HPV prevalence. Prevalence of HPV was 35 % in the saliva of HIV positive patients. High-risk HPV types were more prevalent (26%) than low-risk types (4%). HPV16 was the most prevalent genotype followed by HPV-55 and 83. An increase in oral HPV was noted with increasingly effective HAART.

**Shetty K and Leigh (2006)[52]** described case reports of 4 HIV positive patients who presented with multiple raised papillary lesions on the labial mucosa. These cases suggested a predilection for the labial mucosa for occurrence of oral warts in the HIV positive patients on HAART.

**Fakhry C, D'Souza G, Sugar E *et al* (2007)[37]** evaluated the presence of oral HPV in the oral rinse and cervical vaginal lavage specimens of 123 HIV positive and 59 HIV negative women. These patients were re-examined and samples were again collected after six months and assessed. In HIV-positive women HPV DNA was detected in 24% and 23% of the oral rinse samples and 75% and 70% of cervical vaginal lavage specimens during baseline and follow up examination. This was very much high when compared with oral (8.5% and 13.6%) and cervical (42% and 41%) HPV DNA prevalence among HIV–negative women. The six month cumulative prevalence of oral HPV infection (33%) was significantly less than that of cervical infection (78%). At follow-up, among HIV-positive

women, the rate of oral HPV infections newly detected was significantly lower than cervical infection ($p<0.001$).

**Samples used for HPV detection**

**Zhao M, Rosenbaum E, Koch W*et al* (2005)**[53] to determine the feasibility of using saliva rinses as a screening tool for patients with head and neck cancer in a normal population, the authors studied the presence of HPV 16 in saliva rinse from patients with head and neck squamous cell carcinoma (HNSC) and normal control subjects. Real time quantitative PCR was used to detect HPV 19 DNA in 93 primary tumours and salivary rinses of these patients and 604 control subjects without HNSC. 45.6% of primary HNSC and 32.6% of saliva rinse samples from HNSC patients had HPV whereas only 2.8% of the controls had HPV16 in saliva rinses. Based on these they concluded that salivary rinses have a potential for use in molecular screening for HPV-related HNSC.

**Furrer V E, Benitez M B, Furnes M *et al* (2006)**[54] evaluated the use of a non-invasive technique for HPV genotyping. They compared biopsies and superficial scrapes as sampling methods for the detection of HPV DNA and determination of the HPV genotype spectrum in potentially malignant and malignant oral lesions once clinical features of the lesions were determined. Of the 22 patients with potentially malignant and malignant lesions, 41% of the biopsies were HPV DNA positive, whereas 95–100% of the superficial scrapes were positive. Based on these findings they put

forth that the information on oral HPV infection/oral carcinogenesis depends not only on the DNA detection technique, but also on the tissue/cell sampling procedure.

**Methods of Human Papilloma Virus detection**

**Elamin F, Steingrimsdottir H, Wanakulsuriya S *et al* (1998)**[24] evaluated the role of HPV in oral neoplasms using nested PCR. They analysed oral biopsies collected from 40 patients who were clinically diagnosed as SCCs or potentially malignant mucosal lesions Amplification product was not detected by ethidium bromide staining after the first stage of PCR using MY11/09 primers, whereas, 22 of the 40 samples yielded a detectable PCR product when tagged with radioactive phosphorus ($^{22}P$) and separated on an 8% polyacrylamide gel after the second stage of PCR. HPV 16 and HPV 18 were the only types detected in all tumors by HPV typing.

**Karlsen F, Kalantari M, Jenkins A *et al* (1996)**[55] studied HPV detection in a large series of patients with cervical carcinoma using multiple PCR primers from E1, E6-E7 and L1 lesions of HPV genome. Type-specific primers for HPV 16, 18, 31, 33 and 35 detected more HPV infected patients than the most sensitive consensus primer set, whereas the three consensus primer sets MY 09-11 Gp 5+ - 6+ and CpI-CpIIG, together detected more HPV positive patients than type-specific primers. This suggests that in PCR detection systems, multiple consensus primers and type-

specific primers should be used in order to detect all patients harbouring HPV.

**Harnish D, Belland M, Scheid E *et al* (1999)**[56] compared the five commonly employed PCR consensus primers employed for detection and typing of HPV. Yoshikawa, Manos, Gregoire, van den Brule and Snijders primers (derived from the consensus sequences of L1 or E1 open reading frames) when used to detect HPV DNA in standard HPV DNA-containing preparations, exhibited approximately equal sensitivity.

**Castle P, Schiffman M, Gravit P *et al* (2002)**[57] analysed 2978 cervical cell specimens by MY09–MY011primer set, using Ampli Taq Gold DNA polymerase (MY-Gold) and compared with AmpliTaq DNA polymerase (MY-Taq). They then redesigned a new MY09/11 primer set (PGMY-Gold), and compared with MY-Gold for HPV DNA detection. A good agreement between the two methods and no significant differences in HPV detection was seen. It was found that MY-Gold was a more sensitive assay for the detection of HPV DNA than MY-Taq. They suggested that studies reporting HPV DNA detection by PCR need to report the type of polymerase used, as well as other assay specifications.

**Malijn A, Kleter B, Quint W *et al* (2004)**[58] reviewed the molecular diagnostic methods used to detect and identify HPV. The methods for HPV nucleic acid detection methods include, signal

amplification system, target amplification systems (including type specific and broad spectrum PCR, real time PCR and RT-PCR) and detection and analysis of amplified products (PCR and restriction fragment length polymorphism, hybridization analysis of PCR products, microtiter plate hybridization, direct sequence analysis of PCR products and reverse hybridisation).

**D'Souza G, Sugar E, Ruby W *et al* (2005)**[59] investigated the effect of proteinase K digestion (PKD) and heat inactivation; PKD and ethanol precipitation (EP); PKD, phenol-chloroform extraction, and EP; use of the Puregene DNA purification kit; and use of the QIAamp DNA Blood Midi kit for DNA purification.. Puregene-purified samples had higher human DNA yields and Puregene purification detected the greatest number of HPV-positive subjects and total HPV infections in comparison to the numbers detected by all other methods. They concluded that the method of DNA purification from oral rinse samples has a potentially large impact upon the ability to detect HPV genomic DNA in these samples by PCR amplification.

**Boy S, Van Rensburg E, Engelbrecht S *et al* (2006)**[60] compared the three types of nucleic acid hybridization methods for HPV detection, namely Southern blot, In-situ hybridization (ISH) and hybridization signal amplification to evaluate the prevalence and possible role of HPV in primary OSCC in a South African population. Of the 59 OSCC cases analysed, HPV18 DNA was

detected in the primary tumours of seven patients using real-time PCR, whereas no HPV signals were detected with ISH or amplified ISH techniques. They concluded that HPV is probably unimportant in pathogenesis of OSCC and hypothesize HPV detection techniques as the main reason for the positive results in many studies.

**Castle P, Porras C, Quint W *et al* (2008)[61]** compared two consensus primer PCR genotyping methods one based on the PGMY primers and one based on the SPF10 primers for the detection of individual HPV genotypes and carcinogenic HPV genotypes as a group in the cervical specimen of females. These methods differed in the following ways: DNA extraction and purification, amount of purified DNA amplified, PCR primers used and the method of amplicon detection. They found that both the methods were very similar in the overall detection of carcinogenic HPV but the linear array method detected more HPV16, 18, 39, 58, 59, 66, and HPV68/73, while the SPF10 method detected more HPV11, 31, and HPV52.

10 HIV seropositive patients who were on HAART for six months duration constituted the study Group I. Ten HIV seronegative individuals were included in the study, as control group (Group II). Group I **(Table 1, Graph 1)** comprised of 8 (80%) males and 2 (20%) females. The mean age of the patients in group I was 35 ± 8.95years. Of the 10 Group I patients, there were 4 (40%) patients in 21-30 years age group, 3 (30%) patients in 31-40 years age group and 3(30%) patients in 41-50 years age group. **(Table 2, Graph 3)**

Group II comprised of 4 (40%) females and 6 (60%) males (Table 1, Graph 2). The mean age of the subjects in the group II / control group was 27.6± 6.17 years. Of the 10 Group II patients, there were 7 (70%) patients in 21-30 years age group, 2 (20%) patients in 31-40 years age group and 1(10%) patients in 41-50 years age group **(Table 2, Graph 3)**

The systemic lesions among the patients in group I (n=10) were tuberculosis (n=3), haemophillia (n=1) and hydrocele (n=1), lypodystrophy (n=1), diabetes (n=1).

On intraoral examination, of the 10 patients in group I, 5 (50%) presented with gingivitis or periodontitis, 2 (20%) presented with OC, 2 (20%) presented with increased oral melanin pigmentation, 2 (20%) presented with oral submucous fibrosis and 2 (20%) presented dental caries.

The predominant route of transmission of HIV in group II subjects was heterosexual transmission (n=8, 80%), one patient acquired the infection through homosexual route and another patient through blood transfusion **(Table 3, Graph 4)**

The drug regimen taken by group I subjects was 2NRTI+1NNRTI (N=9, 90%), 1NRTI+PI (N=1, 10%) **(Table: 4, Graph: 5)**

The mean CD4 count of group I subjects prior to the initiation of HAART was 122±73.9cells/cu mm and after six months of initiation of HAART was 311±104.4cells/cu mm, (P=0.000) **(Table 5, Graph 6)**

The mean hemoglobin count of group I subjects prior to the initiation of HAART was 11.97±2.38gm/dl and after six months of initiation of HAART was 13.8±1.46gm/dl, (P=0.024) **(Table 6, Graph 7)**

Saliva samples were collected from all the 10 patients in group I. Scrapings from buccal mucosal were obtained from 5 of the 10 patients in group I, chosen randomly. Saliva samples were collected from all the 10 patients in group II. DNA was extracted from all the samples as per the protocol.

All the samples, along with positive and negative controls were amplified using housekeeping gene and MY09/11 primers to

amplify β-globin and L1 regions in a multiplex PCR. Amplified products were subjected to gel electrophoresis. In each gel the positive control DNA from HeLa cell line and negative control without any DNA were included.

The DNA ladder marker was added, in the first or second wells of each gel, with which the molecular weight was compared.

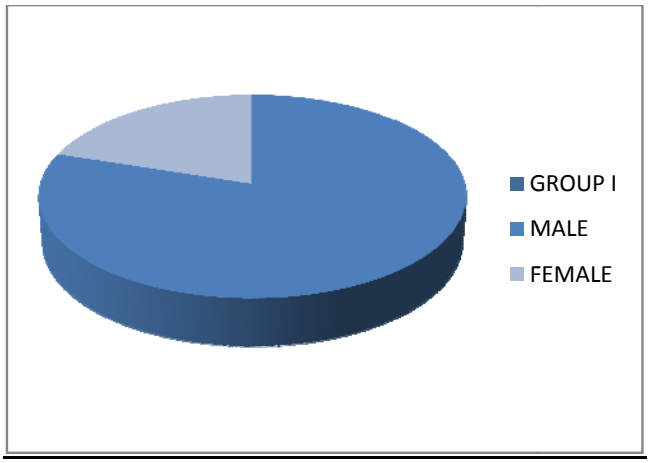
In each gel, the PCR amplification products of all the samples and positive controls showed a clear band for housekeeping gene, β-globin at 250 base pairs (at a level between the marking 200 and 300 on 100 base pairs ladder).

HPV was not detected in any of the samples in both groups **(Figure: 8, 9, 10, 11).** No band was seen corresponding to 450 base pairs size (at a level between the marking 400 and 500 on 100 base pairs ladder) in samples from both groups as well as negative control, though positive control yielded a clear band at 450 base pairs.

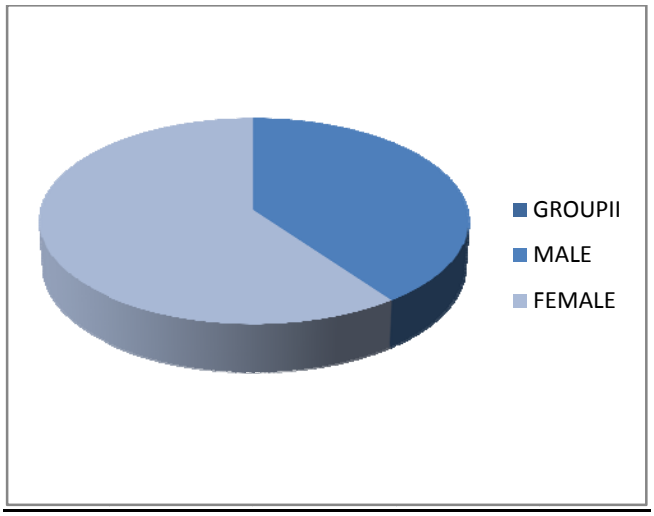
**Table 1: Gender distribution**

| Gender | Group I | Group II |
|---|---|---|
| **Males** | 8 (80%) | 4(40%) |
| **Females** | 2 (20%) | 6 (60%) |

**Graph 1: Gender distribution in group I**




**Graph 2: Gender distribution in group II**



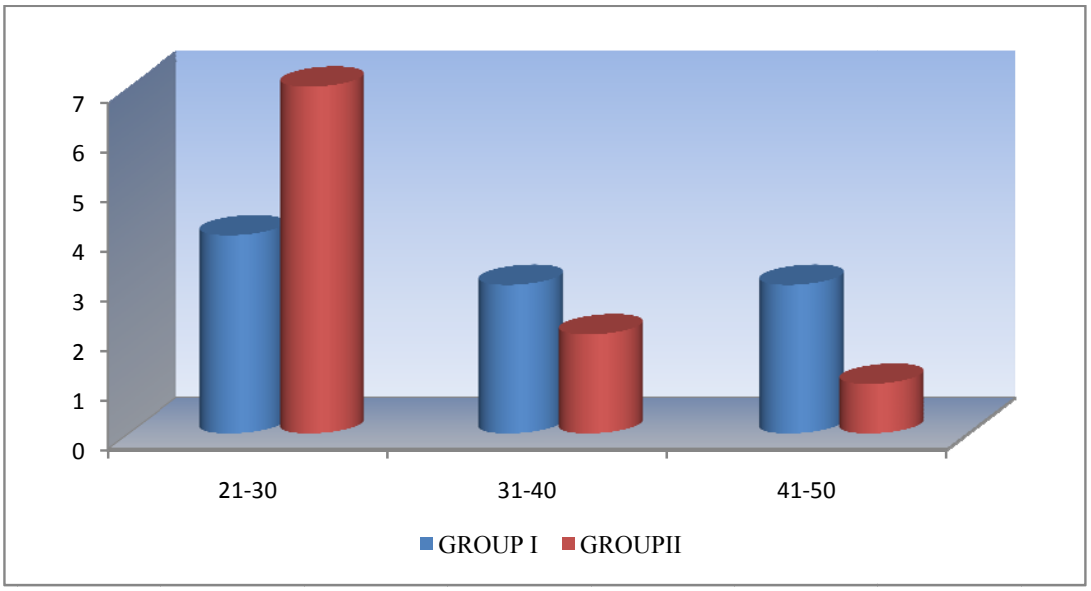

***Group I – HIV seropositive positive individuals ≥ 6 months HAART***

***Group II – HIV seronegative individuals***

## Table 2: Age distribution in the study population

| AGE GROUP | GROUP I N(%) | GROUPII N(%) |
|---|---|---|
| 21-30 | 4 (40%) | 7 (70%) |
| 31-40 | 3 (30%) | 2 (20%) |
| 41-50 | 3 (30%) | 1 (10%) |

## Graph 3: Age distribution in the study population



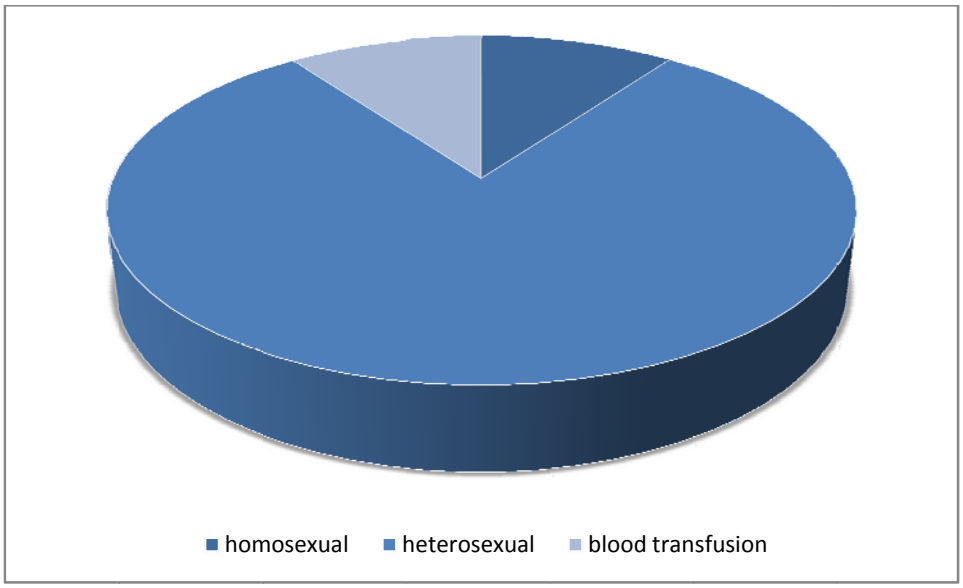

***Group I – HIV seropositive positive individuals ≥ 6 months HAART***

***Group II – HIV seronegative individuals***

**Table 3: Route of transmission of HIV in group I subjects**

| Route | N (%) |
|---|---|
| Homosexual | 1(10%) |
| Heterosexual | 8 (80%) |
| Blood transfusion | 1 (10%) |

**Graph 4: Route of transmission of HIV in group I subjects**

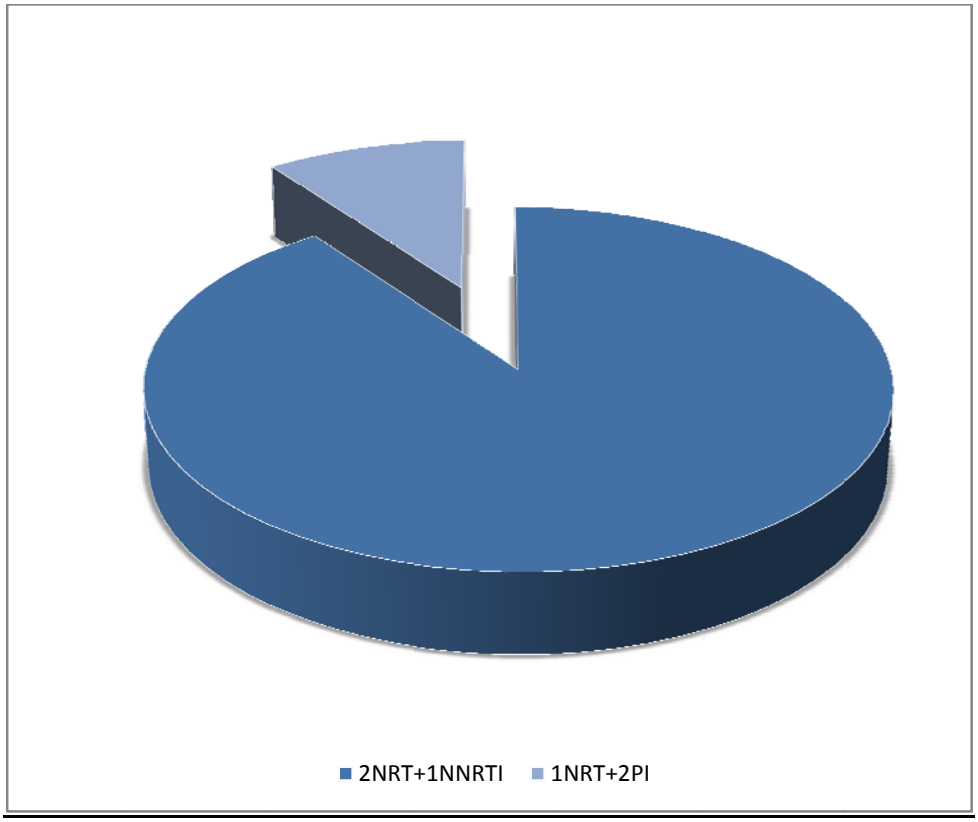



*Group I – HIV seropositive positive individuals( ≥ 6 months HAART)*

**Table 4: Drug regimen taken by patients in group I**

| DRUG REGIMEN | N (%) |
|---|---|
| 2NRTI+1NNRTI | 9 (90%) |
| 2NRTI + 1 PI | 1 (10%) |

**Graph 5: drug regimen taken by patients in group II**




*Group I – HIV seropositive positive individuals ≥ 6 months HAART*

**Graph 6: CD4 count pre and post HAART**

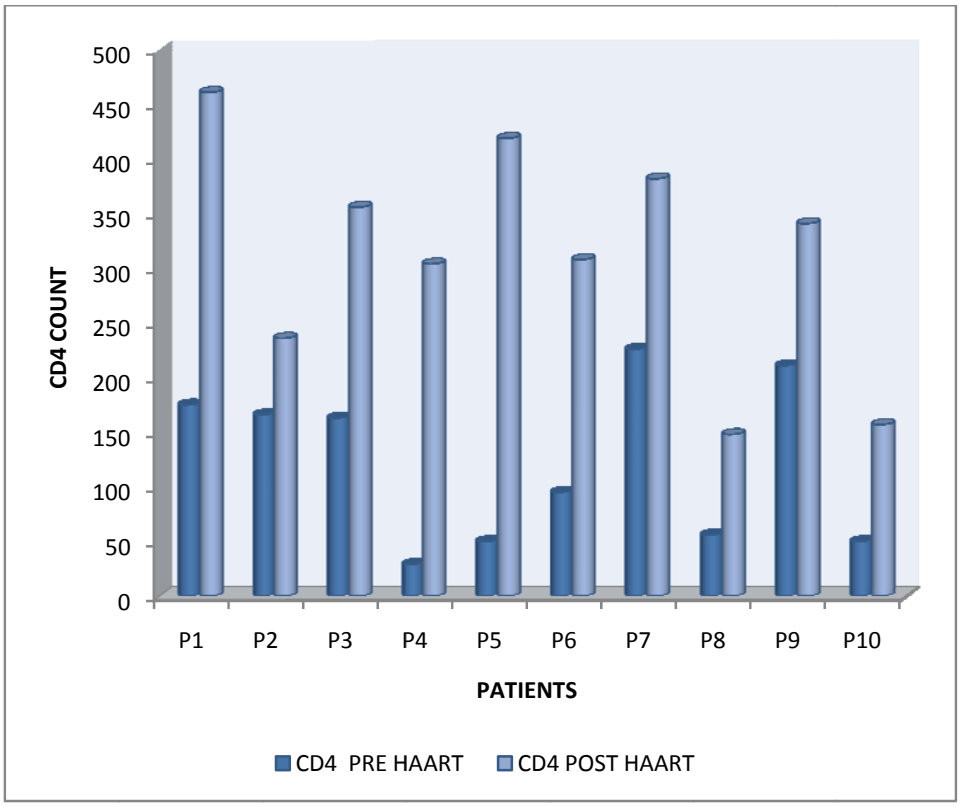
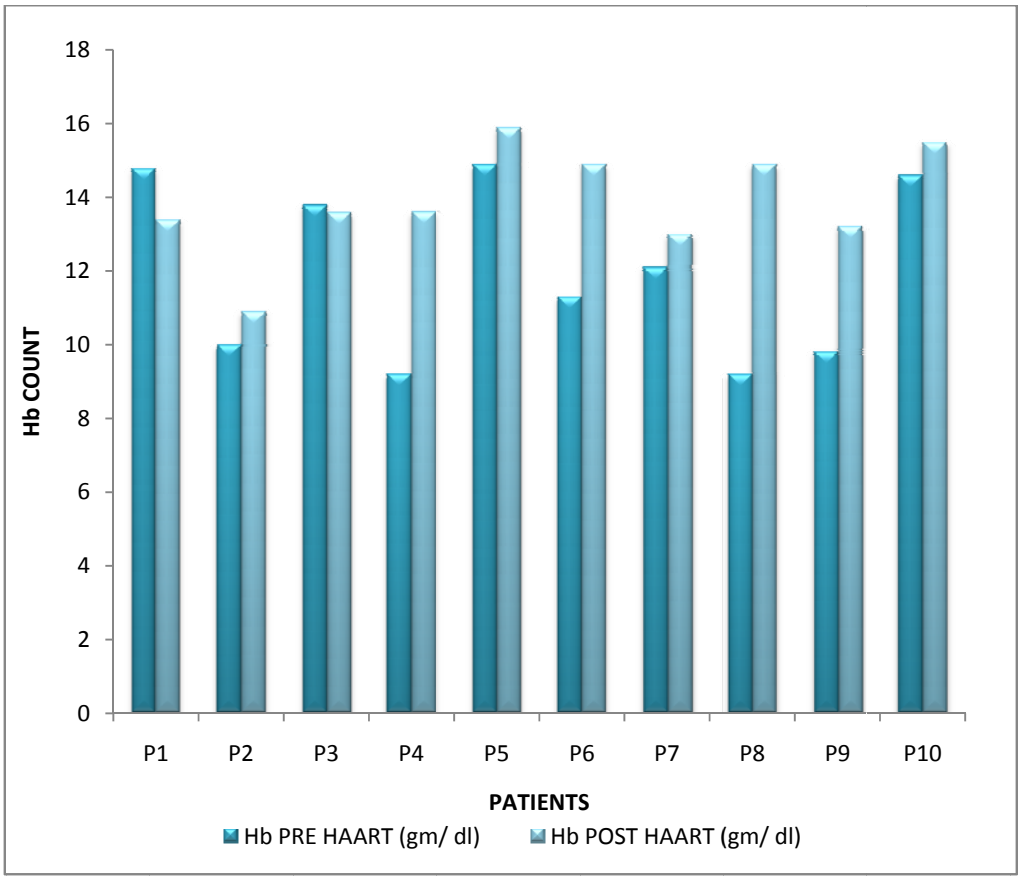



**Table 5: CD4 count pre and post HAART in group I**

| Mean CD4 pre HAART | Mean CD4 post HAART | P value |
|---|---|---|
| **122±73.9 cells/Cu mm** | 311±104.4 cells/Cu mm | 0.00 |

***p value ≤ 0.05 is statistically significant***
***Group I – HIV seropositive positive individuals ≥ 6 months HAART***

## Graph 7: Hb pre and post HAART




## Table 6: Hb count pre and post HAART in group I

| Mean Hb pre HAART | Mean Hb post HAART | P value |
|---|---|---|
| **11.97 ± 2.38gm/dl** | 13.8± 1.46 gm/dl | P = 0.024 |

***p value ≤ 0.05 is statistically significant***
***Group I – HIV seropositive positive individuals ≥ 6 months HAART***

# ARMAMENTARIUM

**Figure 1: DNA Extraction Kit**


**Figure 2: *Pulse vortex***

**Figure 3:** ***Cooling Centrifge***


**Figure 4***: GD column placed inside collecting tube & Micro-centrifuge tube*

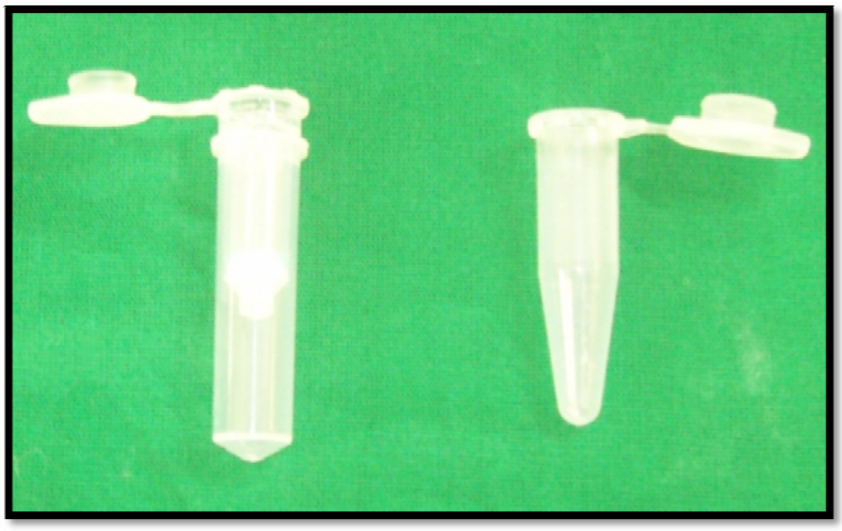


**Figure 5: Automated Thermal Cycler**


**Figure 6: Gel electrophoresis unit**

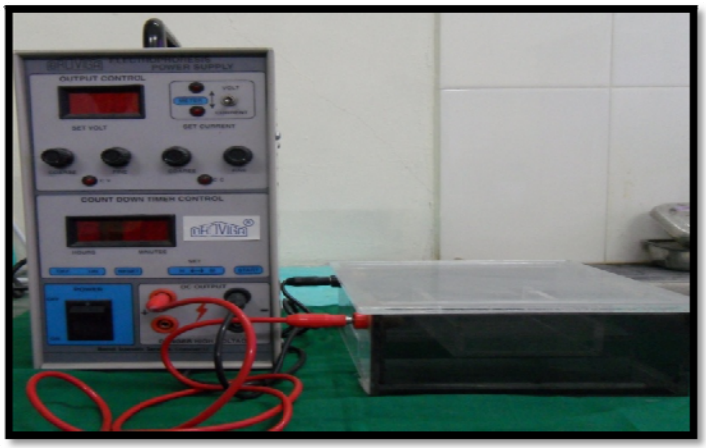
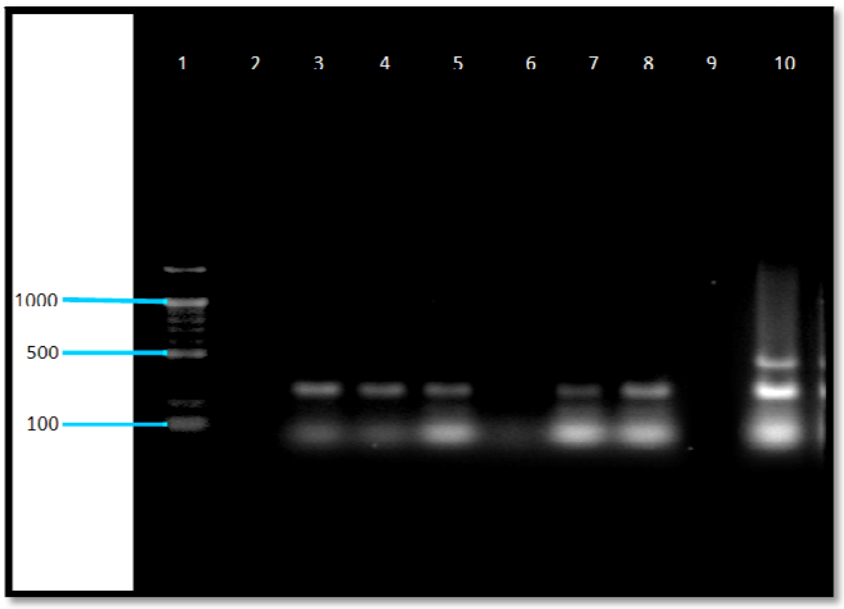



**FIGURE 7 UV – G*el doc system***

**Figure 8: Image of the Gel showing DNA ladder, positive control, samples (P*1& P2, P3, P4,P5) & negative control**




Lane 1 : DNA ladder

Lane 2 : Empty

Lane 3 : Sample P1

Lane 4 : Sample P2

Lane 5 : Sample P3

Lane 6 : Negative control

Lane 7 : Sample P4

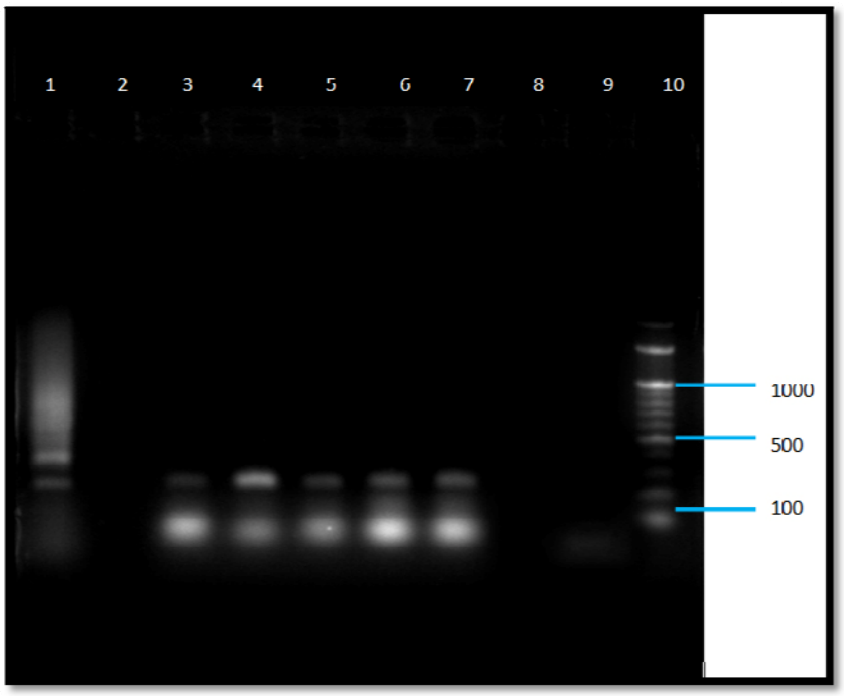
Lane8 : Sample P5

Lane9 :Empty

Lane10 : Positive control (L1 & β globin gene product

**P – HIV sero-positive patients (study group)*

**Figure 9 : Image of the Gel showing DNA ladder, positive control, Samples (P*6& P7, P8, P9,P10) & negative control**




Lane 1 : Positive control (L1 & β globin gene products)

Lane 2 : Empty

Lane 3 : Sample P6

Lane 4 : Sample P7

Lane 5 : Sample P8

Lane 6 : Sample P9

Lane 7 : Sample P10

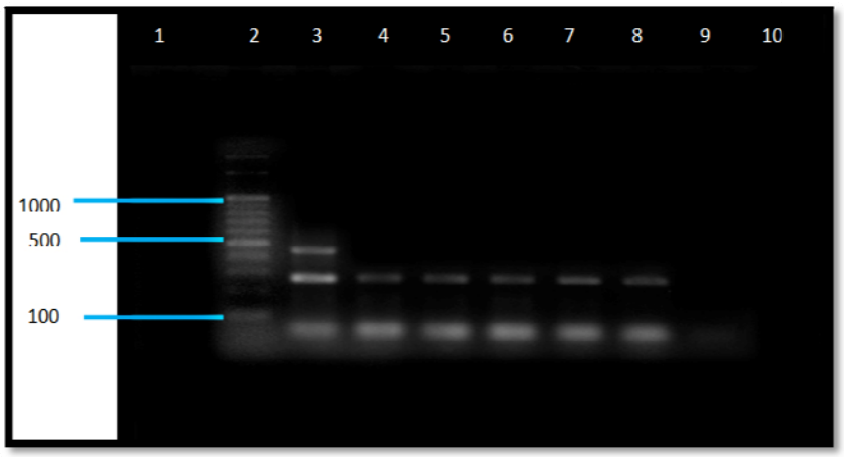
Lane8 : Empty

Lane9 : Negative control

Lane10 : DNA ladder

**P – HIV sero-positive patients (study group)*

**Figure 10: Image of the Gel showing DNA ladder, positive control, Samples (N*1& N2, N3, N4,N5) normal patients**




Lane 1 : Empty

Lane 2 : DNA ladder

Lane 3 : Positive control (L1 & β globin gene products)

Lane 4 : Sample N1

Lane 5 : Sample N2

Lane 6 : Sample N3

Lane 7 : Sample N4

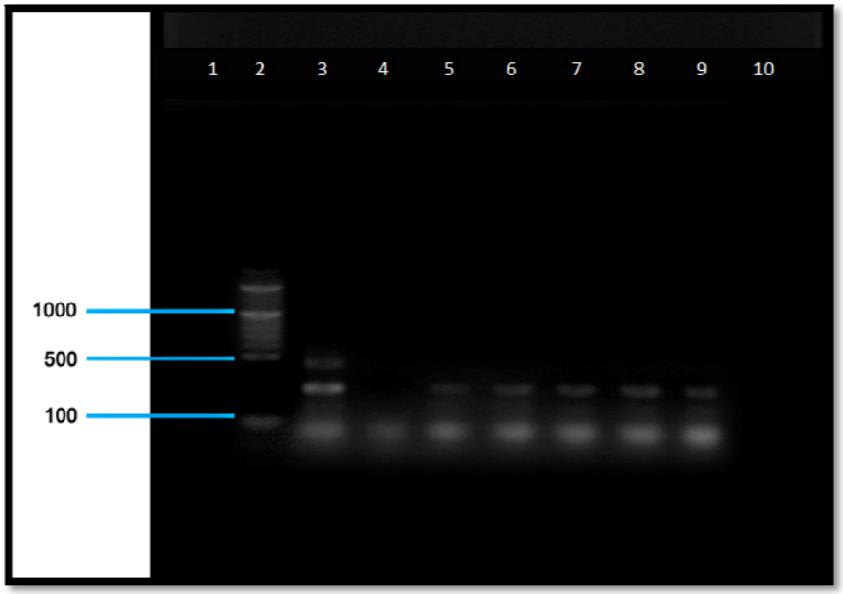
Lane8 : Sample N5

Lane9 : Negative control

Lane10 : Empty

**N – HIV sero- negative patients (study group)*

**Figure 11 : Image of the Gel showing DNA ladder, positive control, samples (N*6& N7, N8, N9,N10) normal patients**




Lane 1 : Empty

Lane 2 : DNA ladder

Lane 3 : Positive control (L1 & β globin gene products)

Lane 4 : Negative control

Lane 5 : Sample N6

Lane 6 : Sample N7

Lane 7 : Sample N8

Lane8 : Sample N9

Lane9 : Sample N10

Lane10 : Empty

**N – HIV sero- negative patients (study group)*

Oral lesions in HIV infection are the earliest and one of the most important indicators of HIV infection. These lesions may be present in upto 50% of patients with HIV infection and in upto 80% of those with a diagnosis of AIDS. These lesions parallel the decline in CD4+ cell counts and an increase in viral load. They are important indicators of disease progression and immunosuppression. Their importance has been demonstrated and emphasized by many researchers[2]

Mortality and morbidity related to AIDS have decreased among HIV infected patients taking highly active antiretroviral therapy (HAART)[5] . A marked reduction in the prevalence of many oral lesions have been reported after the introduction of HAART.

HPV infection has taken on much greater significance in recent years, as the frequency of HPV infection has increased in examined groups following the introduction of HAART and especially, given the fact that this virus may be involved in the etiology of squamous cell carcinoma[8].

Greenspan *et al* (2001) among the 1280 patients referred to their clinic in San Fransisco over a period of 9 years, reported an increase in the prevalence of oral warts in patients on anti-retroviral therapy (15%) and patients on HAART (23%) when compared to patients not on any anti-retroviral medication (5%)[13].

LL Patton *et al* (2000) reported that though the prevalence of oral lesions (hairy leukoplakia, necrotizing periodontal disease) significantly decreased, there was a significant increase in the HIV associated salivary gland disease and human papilloma virus infections. [62]

Green wood *et al* (2002), in their retrospective study of 1590 patients attending the dental clinic at UK, found that there was a marginal increase in the incidence of HPV lesions following HAART with significant decline of other oral lesions. Andrea M *et al* in their study of a German cohort of 103 HIV positive patients also reported a similar findings.[63] Mark D king et al in their study of 2194 HIV positive patients attending the health centre at Atlanta, Georgia also reported that the prevalence of oral HPV increased significantly in the era of HAART, the prevalence and incidence of HPV was 2.6 and 1.6 respectively. [64]

The cause of high prevalence of oral HPV infection in HIV infected individuals is not clear. The high risk sexual practices that are common among HIV infected individuals and multiple exposures may predispose HIV infected individuals to acquire HPV. Alternatively, the higher HPV detection rates could be due to increased HPV replication and/or persistence rather than increased HPV acquisition.[15]

Langerhans cells, which are important for antigen presentation, have been shown to be depleted in the oral mucosa of HIV infected persons. It has also been hypothesized that restoration of langerhan cells and CD4 T lymphocytes occurring in the oral mucosa in response to reduction in viral load allow for a marked improvement for HPV antigen recognition and presentation. Subsequently, local inflammatory responses may also improve, when the inflammatory response become marked, clinically evident oral warts become marked[65].

HIV regulation of HPV transcription is another possible mechanism for the increase risk of oral warts seen in response to significant reduction of viral load. The presence of HIV tat proteins has been found in condyloma accuminatum tissue samples obtained from HIV infected patients for biopsy[66]

It is also postulated that individuals who are on HAART have already been immunocompromised long enough to surpass some critical threshold for developing HPV related disease, which may not be reversed with antiretroviral therapy.[11]

Other reasons for the reported increase in incidence of oral warts could be epidemiological, chemical (direct impact of treatment regimens on the host/virus), viral (interactions between the HIV and HPV) and immunological (lack of restoration of an essential component of HPV immune control)[67]

It is known that clinical lesions are not manifested in all cases of oral HPV infection and diagnosis of sub-clinical infections is easily missed. Hence we used a molecular method (PCR) to detect HPV in our study. Majority of the previous studies in HIV infected patients have focussed on detection of oncogenic HPV-16 and HPV-18 subtypes in the head and neck region, with few reports on other oncogenic and low risk types of HPVs[35,36,37].

We used a consensus L1primer set (MY 09/11) since it amplifies the L1 region of HPV genome which is highly conserved.

HPV has been detected in the saliva and buccal mucosal scrapings previously by Cameron *et al* (2005)[11]. They studied the saliva of 98 HIV positive individuals and detected HPV infection more frequently in patients on effective HAART (57%) compared to those on clinically ineffective HAART (29%). HAART efficacy was defined by a HIV viral load <400 copies/ml in their study. Oral HPV was detected in 37% of HIV positive African American individuals. Caucasians were at greater risk of oral HPV infection than African Americans[11].

Zhao *et al*[53] and Karlsen *et al* (1996)[55] have done similar studies by using MY 09/11 primers and successfully detected the HPV DNA. Both high and low risk HPV types have been detected using L1 consensus primer.

In our study we used RBC spin column based DNA extraction kit to extract DNA from the samples. Both unstimulated saliva and scrapings from buccal mucosa were analysed. All the samples had more than 50ng/µl of DNA. For PCR analysis, 5-10ng of DNA is sufficient. The DNA so obtained was subjected to the PCR amplification and gel electrophoresis, to amplify the housekeeping gene and HPV DNA. All the samples were positive for housekeeping gene, confirming the presence of adequate amounts of DNA for PCR analysis. None of the samples showed HPV positivity, though all the positive controls gave a positive test result. This confirmed that the PCR did not have any inhibitors and the amplification and detection of amplified products was defective.

In our study we did not find any HPV genome positivity following HAART therapy, a finding contrary to that of Cameron et al[11] as they identified HPV genome in those on HAART and not on HAART. In HIV patients on HAART, it was detected more frequently in patients on clinically effective HAART (57%) or marginally effective therapy (54%) compared to those on clinically ineffective HAART (29%). A geographically varied group of Africans, Americans and Caucasians were enrolled in this study.

Oral mucosal abnormalities and more than one oral sex partner and oral sex with men are known to be associated with HPV seropositivity[13]. In our study the patients were predominantly heterosexual (80%) in behaviour with the possibility of reduced

frequency of oro-genital contacts and this could possibly explain the absence of oral warts in our study, whereas in the study by Cameron *et al*, only 14 % of the patients were heterosexual.

The increased risk of oral warts that accompanies reductions in viral load following HAART may represent a form of immune reconstitution syndrome occurring in response to improved cell mediated immune function[64]. It is possible that reconstitution of the immune system may be functionally incomplete and its effectiveness might therefore vary with regard to different pathogenic microorganisms. In particular newly generated CD4 cells are sub optimally immunocompetent, hence permitting replication of HPV and development of oral lesions[9].

Our study was a longitudinal study. The mean follow up duration was ±10.6 months. In our study patients there was a significant rise in the CD4 count suggestive of effective HAART therapy. In the study by Greenspan et al, increased HPV induced wart in the post HAART era was suggested as a feature of immune reconstitutional inflammatory syndrome (IRIS). IRIS manifests in about 6 months of the initiation of HAART and none of our patients clinically or sub clinically possessed any HPV genome[13].

In this study we used L1 consensus primer that amplifies the 450 base pair, and this was considered to be the most sensitive method for detection of HPV. Given the fact that we could not

detect HPV in our patients who were on HAART, we state that there is absence of HPV in the oral cavity of our cohort.

Oral warts occurring in HIV seropositive patients often have an uncharacteristic morphology and may be recalcitrant to therapy and HPV related lesion in HIV disease may be associated with moderate or severe oral epithelial dysplasia[9].

Given these facts we state that there is a need to adopt a screening procedure to determine the likely risk of oral carriage of HPV in order to ensure careful clinical follow up of these patients who are especially on HAART.

- This study was done to detect HPV in the saliva of HIV seropositive patients on HAART therapy (for ateast six months)
- Ten HIV seropositive patients were recruited for the study≥ six months of initiation of HAART (group I) and 10 HIV seronegative subjects were controls (Group II)
- DNA was extracted from all the salivary samples. The DNA was quantified (≥25 µg/dl)
- Extracted DNA was amplified for the identification of HPV using L1 consensus primers and primers to amplify the house keeping gene by multiplex PCR method
- All samples were positive for β globin (house keeping) gene and positive control (infected HeLa cell line DNA) was positive for HPV DNA
- We were unable to detect HPV DNA in our study subjects of both group

We used the most sensitive method, PCR to detect HPV genome in our study subjects. To understand the biology of HPV infection in HIV seropositive patients, more longitudinal studies are necessary especially in the context of HAART.

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**ANNEXURE 1: Demographic details, clinical history, drug regimen and laboratory findings of study group – group I (HIV seropositive patients on ≥ 6 months of HAART)**

| O.P NO | HAART INITIATION | DRUG REGIMEN | CD4 COUNT | CD4 (POSTHA ART) | Hb | Hb (post HAART) | REGIMEN | DURATION OF HAART | AGE | SEX | OCCUPATION | MARITAL STATUS | MODE OF TRANSMISION | SYSTEMIC LESION PRE HAART | SYSTEMIC LESION POST HAART | ORAL LESION PRE HAART | ORAL LESION POST HAART | HPV detection |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M471 | 13.11.09 | EFV+3TC+TDF | 175 | 461 | 14.8 | 13.4 | 2NRT+1N NRTI | 6 | 23 | m | STUDENT | SINGLE | BLOOD TRANSFUSION | HAEMOPHILLIA | HAEMOPHILLIA | GINGIVITIS | GINGIVITIS | NEGATIVE |
| L260 | 19.11.09 | AZT +3TC +EFV | 166 | 236 | 10 | 10.9 | 2NRT+1N NRTI | 7 | 50 | f | HOUSE WIFE | WIDOW | HETEROSEXUAL | NIL | NIL | MELANOSIS | MELANOSIS | NEGATIVE |
| A813 | 19.08.09 | ATV+RTV+EFV | 163 | 356 | 13.8 | 13.6 | 1NRT+2PI | 10 | 43 | m | COOL DRINKS SHOP | MARRIED | HETEROSEXUAL | TUBERCULOSIS | TUBERCULOSIS | GINGIVITIS | GINGIVITIS | NEGATIVE |
| S3173 | 17.9.09 | TDF + 3TC+EFV | 29 | 304 | 9.2 | 13.6 | 2NRT+1N NRTI | 10 | 40 | m | FINANCE BUSSINESS | MARRIED | HETEROSEXUAL | TUBERCULOSIS | TUBERCULOSIS | PERIODONTITIS,ROOTSTUMP | PERIODONTITIS,ROOTSTUMP | NEGATIVE |
| S3220 | 12.08.09 | EFV+3TC+TDF | 50 | 419 | 14.9 | 15.9 | 2NRT+1N NRTI | 14 | 34 | m | AGRICULTURE | SINGLE | HETEROSEXUAL | TUBERCULOSIS | TUBERCULOSIS | PC,GINGIVITIS | GINGIVITIS | NEGATIVE |
| S3106 | 01.10.09 | AZT +D4T +EFV | 95 | 308 | 11.3 | 14.9 | 2NRT+1N NRTI | 8 | 23 | m | LABOURER | SINGLE | HOMOSEXUAL | NIL | NIL | OSMF,PC | OSMF | NEGATIVE |
| Y75 | 11.11.09 | EFV+3TC+D4T | 226 | 382 | 12.1 | 13 | 2NRT+1N NRTI | 10 | 38 | f | LABOURER | MARRIED | HETEROSEXUAL | NIL | UTI | GINGIVITIS | GINGIVITIS | NEGATIVE |
| S3161 | 1.10.09 | EFV+3TC+D4T | 56 | 148 | 9.2 | 14.9 | 2NRT+1N NRTI | 8 | 28 | m | DRIVER | SINGLE | HETEROSEXUAL | HYDROCELE | NIL | DENTAL CARIES | DENTAL CARIES | NEGATIVE |
| K983 | 26.09.09 | AZT+3TC+NVP | 211 | 341 | 9.8 | 13.2 | 2NRTI+1N NRTI | 13 | 41 | m | AGRICULTURE | MARRIED | HETEROSEXUAL | DIABETIC | DIABETIC,LYPHO DYSTROPHY | PC,MELANOSIS | PC,MELANOSIS | NEGATIVE |
| S3211 | 13.09.09 | TDF+3TC+EFV | 50 | 157 | 14.6 | 15.5 | 2NRT+1N NRTI | 12 | 30 | m | SHOP KEEPER | MARRIED | HETEROSEXUAL | PCP | COUGH, BREATHLESSNESS | OSMF | OSMF | NEGATIVE |

# ANNEXURE 2

## Oral HPV Types and Diseases

| Genotype | Diseases |
|---|---|
| HPV 2, 4 | Verruca Vulgaris |
| HPV 6, 11 | Condyloma Acuminatum, Squamous Papilloma |
| HPV 13, 32 | Focal Epithelial Hyperplasia |
| HPV 16 | Proliferative Verrucous Leukoplakia |
| HPV 6, 11, 16 | Verrucous Carcinoma |
| HPV 16, 18 | Squamous Cell Carcinoma |